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The Insulin Family: Evolution of Structure and Function in Vertebrates and Invertebrates

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Abstract. Insulin and related peptides are key hormonal integrators of growth and metabolism in vertebrates. Recently, the amino acid and DNA sequences of insulin-related peptides in invertebrates have become available. The discovery of such peptides in insects and molluscs substantiates the evidence for an early origin and widespread evolution of the insulin superfamily.

In the silkworm *Bombyx* (Insecta) the prothoracicotropic hormones (bombyxins I, II, and III; previously called PTTH) are produced in the brain and may stimulate synthesis and release of ecdysone; thus they play a central role in insect development. In the freshwater snail *Lymnaea* (Mollusca), a growth stimulating hormone (molluscan insulin-related peptide; MIP) is produced in the brain, and two other insulin-related peptides are produced in the digestive system. The MIPs are involved in body and shell growth and energy metabolism. The finding that bombyxin and MIP are involved in the control of growth fits with ideas being developed in the vertebrate field that the role of insulin is not confined to glucose metabolism, but is also related to growth.

Introduction

The structure of the insulin molecule has been highly conserved during vertebrate evolution (Chance *et al.*, 1968; Blundell *et al.*, 1972; Cutfield *et al.*, 1979, 1986; Chan *et al.*, 1981; Bajaj *et al.*, 1983; Emdin *et al.*, 1985; Le Roith *et al.*, 1987; Pollock *et al.*, 1987). At the moment, the primary structures of insulins from over 40 vertebrate species are known. In addition, the preproinsulin genes and cDNA sequences from over 12 species have been determined (Lomedica *et al.*, 1979; Hahn *et al.*, 1983; Steiner *et al.*, 1985). Therefore, for vertebrates, the structures of preproinsulins and the insulin-like growth factors could be integrated to produce an evolutionary picture of this hormone superfamily. We will not discuss the evolution of vertebrate insulins; this has already been done several times (*e.g.*, Steiner *et al.*, 1985). Rather, we will consider the varied evidence, obtained during the last few years, that modern invertebrates, and in particular the insects and the molluscs, also contain insulin-related peptides. Furthermore, we will inquire about the nature of the insulin molecule and compare its functions in insects and molluscs with those in vertebrates.

When considering the evolutionary aspects of insulins in the animal kingdom, we should keep in mind that the various phyla have had a polyphyletic origin; *i.e.*, four major groups—the chordates and vertebrates, the echinoderms and tentaculates, the coelenterates, and the molluscs, worms, and arthropods—are now considered to have evolved independently of each other (Fig. 1). An important consequence of this polyphyletic origin is that if insulin occurs in the two main branches of the phylogenetic tree, then it must have already been present in the Archaemetazoa.

Evidence for Insulins in Invertebrates

Because invertebrates as well as vertebrates rely upon the same organic molecules for metabolism, both groups should, in theory, possess insulin. The experimental evidence in support of this notion comes from two different approaches: immunocytochemistry and biochemistry.

With immunocytochemistry, rapid strides have been made in the identification of invertebrate cells and tissues that are reactive to anti-insulin. Most of the observations have been carried out with antisera raised to mammalian insulin, and positive results have been obtained

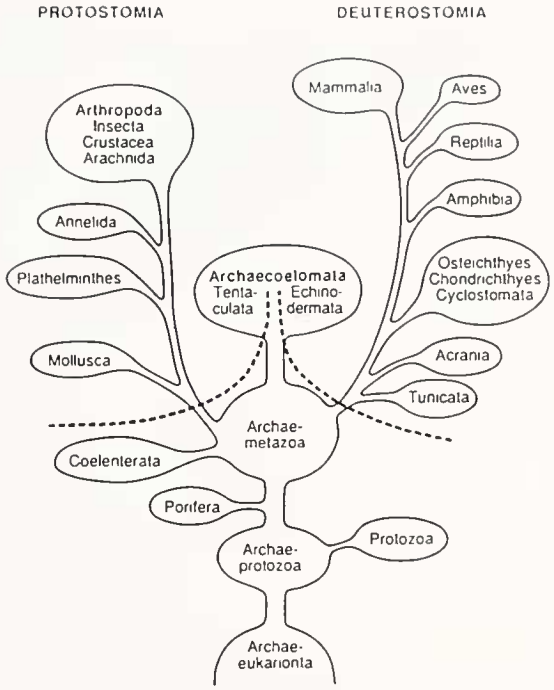

Figure 1. A phylogenetic tree, showing the polyphyletic origin of the various phyla in the animal kingdom. (Modified and extended after Karlson, 1983).

Table I

Identification of insulin-like peptides in invertebrates by immunocytochemistry

Insecta		
Calliphora vomitoria	Median neurosecretory cells	Duve and Thorpe, 1979
Bombyx mori	Median neurosecretory cells	Yui *et al.*, 1980
Locust migratoria	Median neurosecretory cells	Orchard and Loughton, 1980
Manduca sexta	Median neurosecretory cells	El-Salhy *et al.*, 1984
Eristalis aeneus	Pars intercerebralis	El-Salhy *et al.*, 1980
Apis millifera	Brain	Maier *et al.*, 1981
Mollusca		
Lymnaea stagnalis	Small cells in cerebral ganglia	Schot *et al.*, 1981
	Light green cells	van Minnen., 1987
Anodonta cygnea	Midgut	Plisetskaya, 1978
Unio pictorum	Midgut	Plisetskaya, 1978
Mytilus edulis	Hepatopancreas	Fritch *et al.*, 1976
Tunicata		
Steyla clava	Endocrine cells esophagus	Bevis and Thorndyke, 1978

* For references see literature cited in Joosse and Geraerts (1983) and Thorpe and Duve (1984).

in a range of different species, primarily insects and molluscs (Table I). In molluscs, immunoreactivity occurs not only in neuronal tissue, but also in the epithelia of the gut and hepatopancreas.

Of course, there are problems and pitfalls in immunocytochemistry. The epitope for anti-insulin deduced from structure-activity analyses, is formed by the region including residues 8, 9, and 10 of the A-chain, and residues 2, 3, and 4 of the B-chain of insulin. The ability of invertebrate tissues to bind anti-mammalian insulin is surprising because non-mammalian insulins, insulin-like growth factors, and relaxin are variable in this region and, therefore, do not bind antibodies to mammalian insulin. However, the neuroendocrine light green cells in the cerebral ganglia of the central nervous system of the freshwater snail have been identified as anti-porcine insulin immunopositive cells (Fig. 2). Indeed, these cells produce an insulin-related peptide with a different epitope region (see below).

The second approach in the identification of insulin-related peptides is biochemistry: extraction, purification, and chemical characterization. Several early reports of insulin-like substances in invertebrates relied upon rather simple or even crude tissue extraction procedures followed by heterologous bioassays (Table II). Later on, RIA and purification studies were performed. Studies on the blowfly, *Calliphora vomitoria*, by Thorpe and Duve (1984) resulted in the purification and amino acid composition of anti-insulin immunoreactive material, although no amino acid sequence analysis was done. Our recent studies on the snail *Lymnaea stagnalis* have resulted in the structural analysis of an insulin-related molecule from the CNS and the purification of two insulin-like substances from the midgut. We have cleaved and

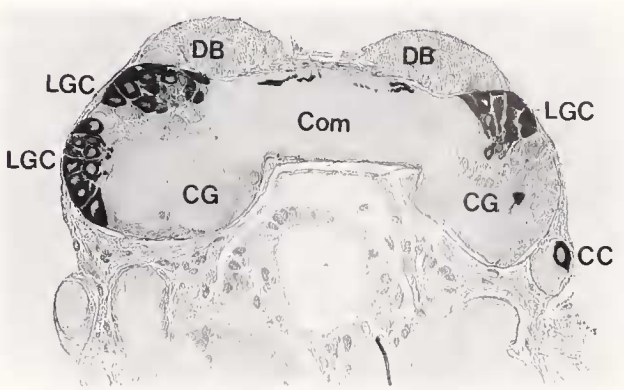

Figure 2. Transverse section through the cerebral ganglia of *Lymnaea stagnalis*. The light green cells (LGC) in the cerebral ganglia (CG) and the canopy cell (CC) in the lateral lobe are labeled by anti-porcine insulin. DB, dorsal body; Com, commissure.

Table II

Biochemical characterization of insulin-like peptides in invertebrates

Insecta		
Apis mellifera	Extract/bioassay	Patel, 1964
Drosophila melanogaster	Extract/bioassay	Meness and Ortiz, 1975
Manduca sexta	Exract/RIA/ bioassay	Kramer *et al.*, 1977
Drosophila melanogaster	Heamolymf/RIA	Seecof and Dewhurst, 1974
Manduca sexta	GPC/RIA	Tager *et al.*, 1975, 1976
Manduca sexta	Amino acid composition	Kramer, 1984
Calliphora vomitoria	GPC/IEG/HPLC	Duve *et al.*, 1979, 1982
	Amino acid composition	Thorpe and Duve, 1984
Drosophila melanogaster	GPC/RIA	Le Roith *et al.*, 1981
Bombix mori	HPLC etc. and sequence	Nagasawa *et al.*, 1984, 1986
Crustacea		
Homarus americanus	RIA/bioassay	Sanders, 1983
Mollusca		
Mya arenaria	extract/bioassay	Collip, 1923
Buccinum imdatum	extract/bioassay	Davidson, 1971
Pectum maximus	extract/bioassay	Davidson, 1971
Ostrea edulis	extract/bioassay	Martinez *et al.*, 1973
Unio pectorum	IEC/RIA	Plisetskaya, 1978
Prophysaon foliolatum	heamolymph/RIA	Plisetskaya and Deyrup, 1987
Lymnaea stagnalis	GPC/HPLC gut-insulin	Hemminga, 1984 Ebberink and Joosse, 1985
Lymnaea stagnalis	HPLC brain-insulin	Ebberink *et al.*, 1987
Lymnaea stagnalis	cDNA brain insulin	Smit *et al.*, 1988
Tunicata		
Pyura pachydermatina	HPLC/RIA	Galloway and Cutfield, 1988

* For references see literature cited in Joosse and Geraerts (1983) and Thorpe and Duve (1984).

separated the A and B chains of the midgut insulin-like substance (Fig. 3), and have determined the amino acid composition of each chain (Table III), but efforts to sequence this material using a protein sequencer have failed since both chains have a blocked N-terminal.

Structure and Function of Insulin-Related Peptides in Invertebrates

The first amino acid sequence information about an insulin-related structure in invertebrates came from the pioneer work of Nagasawa and his colleagues (Nagasawa *et al.*, 1984, 1986) on the prothoracicotrophic hormone (PTTH, now called bombyxin) of the silkworm *Bombyx mori*. Bombyxin is produced by the median neurosecretory cells of the pars intercerebrale of the brain, and controls the secretion of ecdysone from the prothoracic glands during metamorphosis (Ishizaki *et al.*, 1987). Bombyxin was not previously suspected of having any relationship to insulin. The similarity emerged only after 25 years, during which Nagasawa *et al.* purified the peptide from several million heads of *Bombyx* using a 14-step procedure.

Bombyxin II consists of two non-identical chains: the A-chain of 20 residues, and the B-chain of 28 residues (Fig. 4). Besides bombyxin II, two other peptides have been purified. Only the N-terminals of the A-chains of bombyxins I and III have been sequenced, and both have an 80% homology with bombyxin II. Four different forms of bombyxin-II have been published (Fig. 4).

Insulin-related peptides of *Lymnaea* are not only produced in the gut, but also in the neuroendocrine light green cells (LGCs). There are about 200 LGCs located in two pairs of clusters in the cerebral ganglia of the central nervous system of this snail (Fig. 2). The LGC are involved in body and shell growth (Geraerts, 1976; Joosse and Geraerts, 1983; Ebberink and Joosse, 1985). The effects on shell formation include: (1) calcium and bicar-

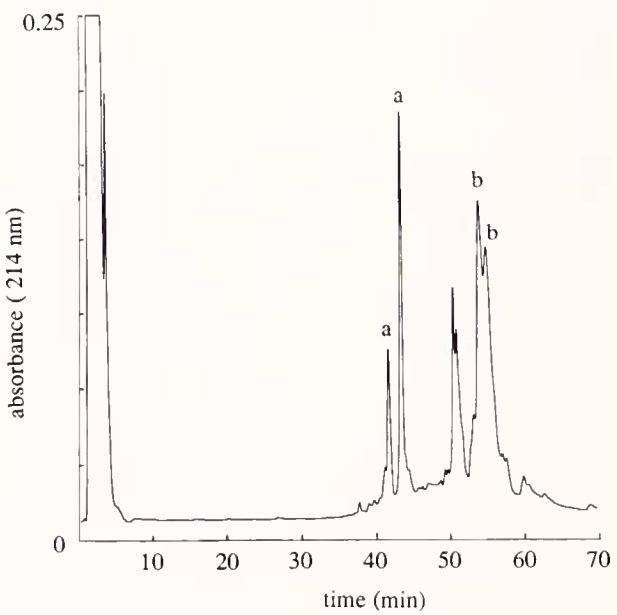

Figure 3. Reverse phase liquid chromatography of purified gut insulin of *Lymnaea stagnalis* after reduction and carboxymethylation. A mixture of two insulin-related peptides was reduced, and the A and B chains were separated on a Bakerbond wide pore C18 column with a gradient of acetonitrile (5–45% in 70 min) in 7.5 m*M* trifluoroacetic acid. The peaks at 51 min are the intact insulins.

Table III

Amino acid composition of the A- and B-chain of gut insulin of Lymnaea *and comparison with brain insulin (MIP) of* Lymnaea *and human insulin*

	A-chain				B-chain			
	Lymnaea gut insulin				*Lymnaea* gut insulin			
	I	II	*Lymnaea* brain insulin	Human insulin	I	II	*Lymnaea* brain insulin	Human insulin
Asx	2	2	1	2	3	3	6	1
Glx	2	2	4	4	2	2	2	3
Cys	~4	~4	5	4	~2	~2	3	2
His	—	1	—	—	3	2	1	2
Ser	2	1	1	2	2	3	5	1
Arg	1	1	1	—	1	1	3	1
Gly	1	1	1	1	2	3	2	3
Thr	1	1	3	1	—	—	—	2
Ala	2	2	—	—	5	6	5	1
Tyr	1	1	1	2	1	1	—	2
Val	1	1	1	1	2	3	3	3
Phe	—	1	—	—	—	—	2	3
Ile	1	1	1	2	2	2	1	—
Leu	2	2	2	2	4	4	2	4
Lys	1	—	1	—	2	2	—	1
Met	nd	nd	1	—	nd	nd	1	—
Pro	nd	nd	2	—	nd	nd	2	1
Trp	nd	nd	—	—	nd	nd	—	—

bonate incorporation into the shell; (2) formation of the periostracum (the proteinaceous component); and (3) the maintenance of high concentrations of calcium-binding protein in the cells of the mantle edge. The effects on the soft body parts are: (1) stimulation of the ornithine decarboxylase activity; (2) mobilization of glycogen stores; (3) regulation of the blood glucose concentration (Fig. 5); and (4) neurite outgrowth.

The first evidence that the LGC may contain an insulin-related peptide came from immunoytochemical data (Fig. 2). To prove that the anti-insulin immunoreactive material is a hormone, the LGCs together with the median lip nerve (the neurohemal area of the LGC), were incubated (*in vitro*) with and without 4-aminopyridine (Fig. 6). After the addition of 4-aminopyridine, the LGC show a strong increase in the number of action potentials, and they release immunoreactive insulin which reaches a maximum level within one hour. In the absence of 4-aminopyridine, only a small amount of immunoreactive insulin was released.

A-chain.

```
 1              5                  10                  15                  20
Gly-Ile-Val-Asp-Glu-Cys-Cys-Leu-Arg-Pro-Cys-Ser-Val-Asp-Val-Leu-Leu-Ser-Tyr-Cys.
```

B-chain.

```
 1              5                  10                  15                  20
Glp-Gln-Pro-Gln-Ala-Val-His-Thr-Tyr-Cys-Gly-Arg-His-Leu-Ala-Arg-Thr-Leu-Ala-Asp-

21             25          28
Leu-Cys-Trp-Glu-Ala-Gly-Val-Asp
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N-terminal region of B-chain of four different forms of Bombyxin-II

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Glp-Gln-Pro-Gln-Ala-Val.....
Glp-Gln-Pro-Gln-Gly-Val.....
Glp----------Gln-Ala-Val.....
Glp----------Gln-Gly-Val.....
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Figure 4. Amino acid sequence of the A and B chains of bombyxin-II (PTTH-II) of *Bombyx mori*.

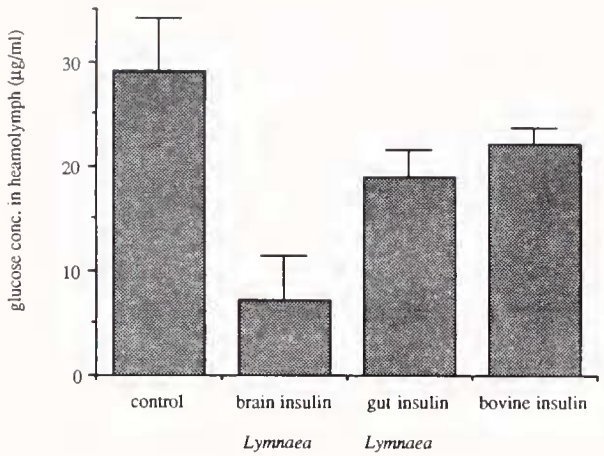
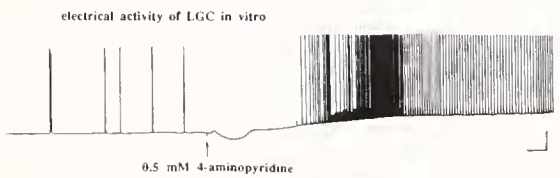


Figure 5. The effect of different insulins on the hemolymph glucose concentration of *Lymnaea stagnalis*. Purified insulins from the light green cells and gut, as well as commercial bovine insulin, were tested. The amount of brain insulin injected in each snail is about the amount stored in one animal (about 2 pmol); for gut insulin, it is the amount stored in about 0.2 animal (about 0.5 pmol); and for bovine insulin, 200 pmol. The blood volume is about 1 ml. (n = 4).

The primary structure of the insulin-related peptide was not obtained via peptide chemistry, but via the nucleotide sequence of an LGC specific cDNA clone (Smit *et al.*, 1988). A differential hybridization technique was used to isolate cerebral LGC cDNA from a central nervous system-specific library of *Lymnaea* cloned in λgt10. Therefore, replica filters of 20,000 clones were screened with a positive cDNA probe synthesized from messenger RNA of the LGC, and with a negative cDNA probe produced from other parts of the cerebral ganglia and the hepatopancreas. The LGC specificity of the clones was tested by *in situ* hybridization using histological sections of the central nervous system (Fig. 7). The LGCs in the cerebral ganglia, and the canopy cell in each lateral lobe (not shown), were the only cell types to express this clone.



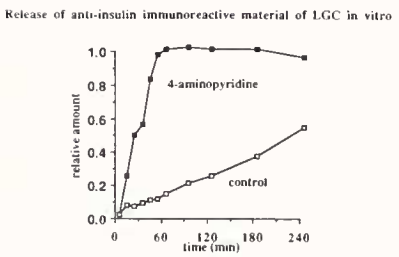


Figure 6. The effect of 4-aminopyridine on the electrical activity of the light green cells (LGC) (top panel). The release of anti-porcine immunoreactive material from the light green cells (bottom panel). Cerebral ganglia (CG) with the median lip nerves were incubated as described previously, with and without 4-aminopyridine (Ebberink *et al.*, 1987).

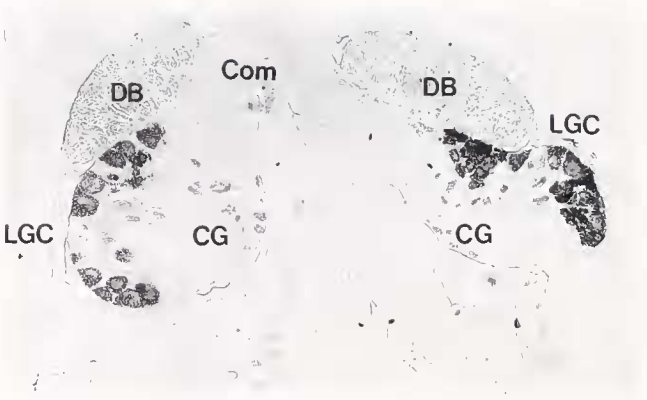


Figure 7. *In situ* hybridization with a 35S-labelled cDNA-probe in sections of the cerebral ganglia. Only hybridization of mRNA in the light green cells (LGC) and canopy cell (not shown) was observed. DB, dorsal body; CG, cerebral ganglia; Com, commissure.

The nucleotide sequence revealed a single open reading frame encoding a protein with characteristics of preproinsulin (Fig. 8) (Smit *et al.*, 1988). Thus, an A and B chain, together with a C peptide equivalent and a putative signal sequence, are present. We called this peptide molluscan insulin-related peptide (MIP).

Comparison of Invertebrate and Vertebrate Insulins

Overall, the amino acid sequences of MIP, bombyxin, and human insulin, are not very similar (Fig. 9). The se-

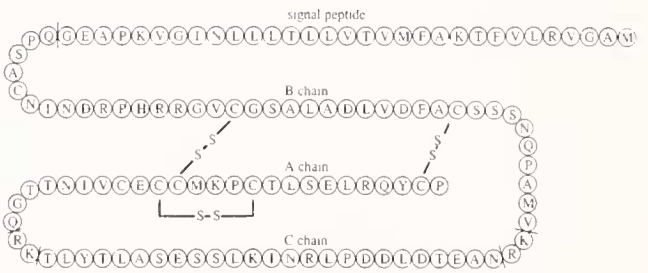


Figure 8. Amino acid sequence of prepro molluscan insulin-related peptide (preproMIP). Residues are designated by their one-letter abbreviations. The putative proteolytic processing sites are indicated (lines between some residues).

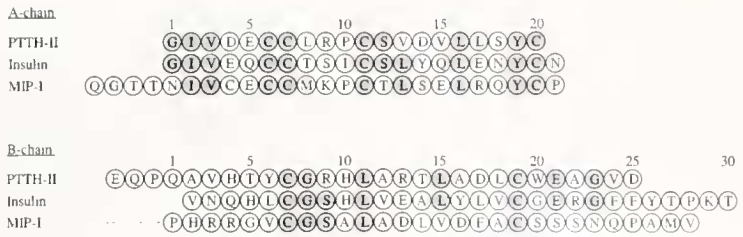


Figure 9. Comparison of the amino acid sequences of the A and B chains of bombyxin (PTTH) of *Bombyx mori*, human insulin, and molluscan insulin-related peptide (MIP) of *Lymnaea stagnalis*. The amino acids are identified by their one-letter abbreviations.

quence similarity in the A chain is about 50% between bombyxin and human insulin, and 40% between MIP and human insulin. In the B chain, the similarity is 25% and 20%, respectively. An amino acid sequence comparison of MIP and bombyxin with known insulins of all vertebrate species permits some interesting conclusions. All of these peptides have cysteines present at position A6, A7, A11, A20, B7, and B19. They have glycine at A1 (except MIP) and at B8, but glycine at B20 is only present in the vertebrate insulins. Most of the hydrophobic residues at the hydrophobic core of the globular structure of insulin are conserved in all insulins. A three-dimensional model of bombyxin and MIP has been constructed with interactive computer graphics and energy minimization techniques (Blundell *et al.*, 1972); homology with porcine insulin (the structure of which has been determined by X-ray analysis) was assumed. The model shows that both bombyxin and MIP form neither the dimers nor the hexamers characteristic of mammalian insulins. The region formed by residues B9-B19 and B22-B26 is involved in the binding of insulin to its receptor (Pullen *et al.*, 1976), and the phenylalanine in position B25 is particularly essential (Blundell and Wood, 1975). Since this region, including B25, differs from that of mammalian insulins, it has been suggested that MIP and bombyxin cannot bind to the vertebrate insulin receptor. However, gut insulin of *Lymnaea* binds very well to insulin receptors of rat fat cells (Ebberink and Joosse, 1985).

Conclusions

It is important that the structures of molluscan and insect insulins have been found at last, more than 30 years after the discovery of the first vertebrate insulin structure.

The structure of the insulin molecule is largely determined by a characteristic arrangement of certain residues in its precursor. That similar arrangements of amino acids seen in the various insulins would have arisen independently in different branches of the phylogenetic tree (Fig. 1) is extremely improbable. We cannot deduce the evolutionary pathway for insulin on the basis of nucleotide differences, since these arise predominantly from neutral mutations. Rather, the finding of insulins in two branches of the phylogenetic tree confirms the model of Blundell and Wood (1975), according to which the evolution of insulins is determined mainly by adaptive processes. The model depends critically on the relationship between such factors as the effects of sequence changes on the three-dimensional structure of the peptide, and the role of various parts of this structure in the conversion of the proinsulin to the active form, the storage of insulin, its transport to the site of action, and its interaction with the receptor. According to this hypothesis, the primary and three dimensional structures conserved in vertebrate insulins must also be conserved in the related peptides of insects and molluscs. Since MIP, the vertebrate insulins and possibly bombyxin are involved in growth, it is important to discover whether the insulin receptors of invertebrates are homologous with those of vertebrates.

According to currently accepted theory, the origin of insulin is to be found in the nervous systems of early multicellular organisms (Pearse, 1967; Pictet *et al.*, 1976; Alpert *et al.*, 1988). Indeed, the localization of an invertebrate insulin within specific neurons of the brain would seem to support such a notion. Until now, the only insulin sequences available were from the central nervous systems of invertebrates and from the pancreas of vertebrates—data that do not test the theory. But we have shown that insulins are present in the gut of molluscs, as well as in the brain. This finding suggests that, contrary to dogma, the insulins might have originated in the brain or in the gut, and possibly also in other tissues of early metazoans. Further, these considerations could imply that the brain of vertebrates also produces insulins. However, at present, the question of the synthesis of insulin in the vertebrate brain is still arguable (Baskin *et al.*, 1987; LeRoith *et al.*, 1987).

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